53.301-298 Standard Form 298, Report Documentation Page.

AD-A280 466

DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	5/22/94	Annual Technical 4/1/93-3/31/94

4. TITLE AND SUBTITLE
Electrophysiological and ionic properties of intrinsic circadian pacemakers in the vertebrate pineal gland.

5. FUNDING NUMBERS
F-49620-93-1-0303
2312/CS
61102F

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8. PERFORMING ORGANIZATION REPORT NUMBER
AFOSR-TR- 94 0356

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Air Force Office of Scientific Research
Life Sciences Directorate
Bolling AFB, DC
20332-6448
Dr Haddad

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited.

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 words)

Cyclic GMP-activated channels of the chick pineal gland are not altered by physiological concentrations of cytoplasmic Ca2+ ions. They are partially blocked by physiological levels of Mg2+. Changes in intracellular pH over a range of 6.2-8.2 do not affect the gating of these channels. Chick pineal cells exhibit spontaneous oscillations in intracellular free Ca2+ and can mobilize intracellular Ca2+ stores. Agents that increase intracellular cyclic AMP cause increases in intracellular Ca2+. Similar effects are caused by VIP but not norepinephrine. Depletion of intracellular stores causes release of a message that promotes influx of Ca2+ from the outside. Internal stores of Ca2+ represent a potential target for the intrinsic circadian oscillator. Inhibition of phosphodiesterases cause activation of cyclic GMP-activated channels in the whole pineal cell, suggesting that phototransduction cascades similar to those of the vertebrate retina are also present in chick pineal cells. A second large-conductance cation channel has also been detected an may play a role in spontaneous or drug-induced Ca2+ oscillations.

14. SUBJECT TERMS	15. NUMBER OF PAGES
	7
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
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A. Research objectives.

1. What are the functions of voltage-evoked ionic currents in isolated chick pineal cells? Are the electrical properties of these cells regulated by neurohormones, second messengers, or the circadian cycle?

2. Do hormones or second messengers regulate intracellular Ca^{2+} concentration? Is there a free-running circadian rhythm in intracellular free Ca^{2+} concentration or sensitivity to extrinsic hormones in cultured chick pineal cells?

3. What are the ionic and circadian mechanisms controlling phototransduction in cultured chick pineal cells?

B. Progress.

Significant progress has been made towards achieving the research objectives described above. Results are summarized below.

1. Properties of cyclic GMP-activated channels in the chick pineal gland: Effects of divalent cations, pH, and cyclic AMP.

These results pertain to research objective 3. These studies have examined whether the gating of cyclic GMP-activated channels is affected by changes in the concentration of cytoplasmic ions known to affect many aspects of the cellular physiology of pineal cells and many other cell types. Results indicate that exposing the cytoplasmic face of the patch membrane to micromolar concentrations of Ca^{2+} ions had no effect on the gating of these channels. The probability of finding channels in the open state and the unitary conductance of the channels was not affected. This is different from olfactory receptor neurons, where micromolar Ca^{2+} is known to decrease the open probability of the cyclic nucleotide-gated channels. In olfactory cells, this effect of Ca^{2+} is thought to play a role in desensitization in the response to odorants. A similar mechanism appears to not take place in the chick pineal gland. By contrast, millimolar concentrations of either Ca^{2+} or Mg^{2+} cause a direct blockade of the channels. This blockade is voltage dependent, and our results suggest that Ca^{2+} and Mg^{2+} block the pores by binding to different sites on the channel molecules. This is also seen in retinal cells. In the pineal, partial blockade of cyclic GMP-activated channels was observed at physiological concentrations of cytoplasmic Mg^{2+}. This suggests that the cyclic GMP-activated channels are partially blocked under normal physiological conditions. The effect of this is to reduce the effective unitary conductance of the channels. A similar situation is found in retinal photoreceptors, and it is generally held that this serves to increase the signal to noise ratio of light-induced

electrophysiological responses. The reason is that normal fluctuations in the number of open channels produces much less fluctuation in the macroscopic responses when the current flowing through any one channel is reduced. One would expect that this would be especially important in small cells such as chick pineal cells, where the resting cell resistance is very high. These studies also found that the gating of cyclic GMP-activated channels is not affected by changes in the cytoplasmic pH over a range of 6.2-8.2 This is important because changes in intracellular pH are known to affect the circadian pacemaker of basal retinal neurons of *Bulla*. The studies also showed that cyclic GMP-activated channels were not affected by physiological concentrations of cyclic AMP. However, very high concentrations of cyclic AMP (~5 mM) caused weak activation of the channels. These high concentrations of cyclic AMP also caused inhibition of gating evoked by physiological concentrations of cyclic GMP, indicating that cyclic AMP functions as a weak partial agonist. Also, in the presence of 5 mM cyclic AMP, channel openings caused by cyclic GMP were often to a subconductance state rarely seen in the absence of cyclic AMP. These results are of biophysical and pharmacological interest, as they are consistent with recent kinetic models proposed for to explain the gating of cyclic GMP-activated channels. But these results probably do not reflect mechanisms that occur in the intact cell, and therefore they will not be pursued further. The studies described above have now been published (Dryer, S. E. & Henderson, D. Cyclic GMP-activated channels of the chick pineal gland: Effects of divalent cations, pH, and cyclic AMP. *Journal of Comparative Physiology A* 172: 271-279).

B. Regulation of intracellular free Ca^{2+} in chick pineal cells.

These results pertain to research objective 2. Fura-2 imaging techniques were used to determine some of the mechanisms that regulate intracellular free Ca^{2+} concentrations in chick pineal cells. As described in the original application, this is important because Ca^{2+} is known to interact with cyclic AMP in the regulation of melatonin synthesis and secretion. Several novel results were obtained in these studies. First, about 10% of cells examined displayed spontaneous oscillations in intracellular free Ca^{2+}. In other types of cells, Ca^{2+} oscillations are often associated with mobilization of intracellular Ca^{2+} stores. Pharmacological studies indicated that chick pineal cells also contain intracellular Ca^{2+} stores. Thus, application of thapsigargin, which mobilizes intracellular Ca^{2+} stores, caused increases in intracellular free Ca^{2+} in chick pineal cells that could be evoked in the *absence* of external Ca^{2+}. This is a very significant finding, as this is the first demonstration of intracellular Ca^{2+} stores in pineal cells of any species. Intracellular Ca^{2+} stores therefore represent a potential output pathway for the intrinsic circadian oscillator in these cells. In the absence of external

Ca^{2+}, responses to thapsigargin gradually subsided owing to depletion of internal stores. Subsequent application of external Ca^{2+} caused a large increase in intracellular free Ca^{2+} that invariably exceeded the peak response to thapsigargin. Similar results have been reported in some other cell types, and the present results suggest that store depletion causes release of a soluble messenger that stimulates Ca^{2+} influx from the outside. Consistent with previous demonstrations of voltage-activated Ca^{2+} channels in chick pineal cells, application of depolarizing concentration of KCl also evoked increases in intracellular free Ca^{2+}, but these responses were entirely dependent upon external Ca^{2+}. Application of the neurohormone VIP, which stimulates cyclic AMP formation and melatonin secretion from these cells also caused in increase in intracellular free Ca^{2+}. In some cells, VIP evoked irregular Ca^{2+} oscillations that persisted for several minutes after the VIP was removed. By contrast, application of norepinephrine, which inhibits cyclic AMP formation and melatonin secretion from chick pineal cells, had no effect on intracellular Ca^{2+} in quiescent cells and did not inhibit Ca^{2+} oscillations in cells where they are present. Because cyclic AMP is known to stimulate melatonin synthesis and secretion, and because VIP stimulates cyclic AMP formation, the effects of agents that increase cyclic AMP on intracellular free Ca^{2+} were examined. Application of the phosphodiesterase inhibitors IBMX or papaverine each evoked increases in intracellular free Ca^{2+}, and, in some cells, evoked Ca^{2+} oscillations. These effects were mimicked by 8-Br-cyclic AMP, a membrane-permeable analog of cyclic AMP, and by forskolin, an activator of adenylate cyclase. The effects of forskolin were observed in the absence of external Ca^{2+} or in the presence of nifedipine, a drug that blocks L-type Ca^{2+} channels. This suggests that cyclic AMP can either directly or indirectly cause mobilization of intracellular Ca^{2+} stores. Martin Zatz has previously shown that Ca^{2+} can cause increases in intracellular cyclic AMP. Therefore, each of these messengers can regulate the intracellular concentrations of the other. This can be regarded as a major advance in the understanding of the cellular physiology of the chick pineal gland. The effects of forskolin were in many ways similar to those of thapsigargin. Thus, the responses to forskolin gradually subsided in the absence of external Ca^{2+}, probably owing to depletion of intracellular stores. A subsequent restoration of external Ca^{2+} caused a sharp increase in intracellular free Ca^{2+} that invariably exceeded the peak response. This again is consistent with release of a messenger by depletion of intracellular stores. This unidentified messenger can then allow for Ca^{2+} influx, probably by pathways *other* than the L-type Ca^{2+} channels. Such a messenger represents a potential output pathway for the circadian oscillator in chick pineal cells. These results were recently accepted for publication in (D'Souza, T. & Dryer, S. E. Intracellular free Ca^{2+} in dissociated cells of the chick pineal gland: Regulation by membrane depolarization, second

messengers, and neuromodulators, and evidence for release of intracellular Ca^{2+} stores. *Brain Research* in press).

C. Effects of phosphodiesterase inhibitors on cyclic GMP-activated channels in intact chick pineal cells, and characteristics of a novel cationic channel.

These results pertain to Objective 3. If phototransduction cascades similar to those of the vertebrate retina are also present in chick pineal cells, then inhibition of phosphodiesterase (which increases both cyclic AMP and cyclic GMP) should cause activation of these channels in intact cells. But forskolin, which causes increases only in cyclic AMP, should not cause activation of these channels. This was addressed using the cell-attached configuration of the patch clamp recording technique. This configuration allows one to record channel activity while maintaining the cytoplasmic face of the patch membrane in contact with the normal cellular cytoplasm. The external saline of the patch pipette was free of divalent cations to improve resolution of the single channel currents. Under these conditions, application of either IBMX or papaverine caused a large increase in the activation of a non-specific cationic channel with a unitary slope conductance of 10-15 pS. This conductance was what would be expected for a cyclic GMP-activated channel in the face of normal physiological concentrations of cytoplasmic Mg^{2+} and in the absence of external divalent cations. Subsequent excision of the patch, thereby removing the cytoplasmic face of the patch membrane from the normal cytoplasm, caused these channels to immediately become quiescent. This suggests that the activation of these channels was caused by some soluble component of the cytoplasm, and was not due to some membrane-associated molecule or by phosphorylation of the channels. By contrast, application of forskolin or 8-Br-cyclic AMP did not cause activation of these channels. These results suggest that cyclic GMP-activated channels can indeed become active upon inhibition of phosphodiesterase, as occurs in vertebrate retinal photoreceptors. This provides additional evidence that the mechanisms of phototransduction are similar in the chick pineal and in rods and cones. In the course of these studies, a second cationic channel was observed that had not been described previously. This channel had a very high conductance (~200 pS) and was a non-selective cationic channel permeable to Ca^{2+}. Its activity was not voltage-dependent and was not affected by drugs that change intracellular cyclic AMP or cyclic GMP. This channel was not active in every cell and was not seen in every patch. But the impact of these channels on these cells could be very significant, as its high conductance could cause large changes in membrane potential and intracellular Ca^{2+} in small chick pineal cells where these channels are active. We are currently characterizing these channels in more detail. A manuscript describing these results is being prepared for publication (D'Souza, T. & Dryer, S. E., Effects of phosphodiesterase inhibitors and

forskolin on the gating of cationic channels in dissociated cells of the chick pineal gland. To be submitted to *Neurochemistry International.*

D. Experiments planned for the coming year.

Methods have now been developed to allow these results to the next stage. All of the apparatus necessary to perform phototransduction experiments on chick pineal cells has been assembled. This entailed setting up an infrared light source and infrared sensitive video camera to allow electrophysiological experiments to be performed under infrared illumination thereby preventing bleaching of the retinal-based photopigments. All sources of stray visible light needed to be eliminated. Apparatus to allow delivery of computer-controlled photic stimuli has also been constructed. Phototransduction experiments will begin later this summer. On the basis of biophysical arguments made by Ed Pugh and Trevor Lamb for retinal cells, it is anticipated that light pulses will hyperpolarize the membrane of chick pineal cells, but that the timecourse of these responses will be considerably slower than in retinal cells.

It is now possible to examine whether the cyclic GMP-activated channels of chick pineal cells are regulated by phosphorylation, especially by cyclic AMP-dependent protein kinase. A new graduate student, Kenneth Mann, will join the laboratory next month, and will undertake these studies. These experiments are straightforward and all necessary techniques are already routine in my laboratory.

Electrophysiological studies are now underway to follow up the results of the Ca^{2+} imaging experiments and to determine if VIP, norepinephrine, or forskolin produce consistent effects of voltage-evoked ionic currents in chick pineal cells.

A cell culture incubator has been modified with lights and timers for photic entrainment of chick pineal cells. This opens up a wide range of experiments that can be performed on cells at different times of day, and, more interestingly, in free-running cells after entrainment. High priority studies include determining whether the expression of voltage- or Ca^{2+} - dependent K^+ channels and Ca^{2+} channels are present at different amplitudes as a function of time of day in free running chick pineal cells, and to determine if resting Ca^{2+} levels and or the proportion of cells showing Ca^{2+} oscillations exhibit free-running circadian rhythms.

C. Publications.

D'Souza, T. & Dryer, S. E. (in preparation) Effects of phosphodiesterase inhibitors, forskolin, and thapsigargin on the gating of cationic channels in

dissociated cells of the chick pineal gland. To be submitted to ***Neurochemistry International***

D'Souza, T. & Dryer, S. E. (1994). Intracellular free Ca^{2+} in dissociated cells of the chick pineal gland: Regulation by membrane depolarization, second messengers, and neuromodulators, and evidence for release of intracellular Ca^{2+} stores. ***Brain Research*** in press.

Dryer, S. E. & Henderson, D. (1993). Cyclic GMP-activated channels of the chick pineal gland: Effects of divalent cations, pH, and cyclic AMP. ***Journal of Comparative Physiology A*** **172**: 271-279.

D. Personnel.

Dori Henderson (now a Ph.D. student at the university of Minnesota Medical School Department of Physiology.

Sanja Raucher, M. D.

Theresa D'Souza (Ph.D. candidate in my laboratory).

Kenneth Mann (Ph.D. student beginning in June, 1994).

E. Coupling activities.

Abstract submitted to the Society for Neuroscience Annual Meeting, Miami FL, 1994.

Invited Lecture, University of Connecticut health Sciences Center, April, 1994.

I have been invited to deliver a major Invited Lecture at the International Eye-Pineal Relationships Symposium to be held September 22-27 in Lodz, Poland.

F. New discoveries and other information.

The finding that chick pineal cells exhibit spontaneous Ca^{2+} oscillations and can mobilize intracellular Ca^{2+} stores represents a major advance in the understanding of the cellular physiology of the chick pineal, as this represents a possible output pathway of the intrinsic circadian oscillator. This research has also provided further evidence that the mechanisms of phototransduction in an intrinsic light-sensitive circadian oscillator are similar to what is observed in the vertebrate retina.